Title: Dosing-time dependent effect of dexamethasone on bone density in rats

Masaki Takahashi, Kentarou Ushijima, Yohei Hayashi, Tomohiro Maekawa, Hitoshi Ando, Shu-ichi Tsuruoka, Akio Fujimura

Division of Clinical Pharmacology, Department of Pharmacology, Jichi Medical University, Tochigi, Japan (M.T., K.U., Y.H., T.M., H.A., and A.F.); Department of Nephrology, Institute of Clinical Sciences, University of Tsukuba, Ibaraki, Japan (S.T)

Address: Division of Clinical Pharmacology, Department of Pharmacology, Jichi Medical University, Tochigi 329-0498, Japan

Phone: +81-285-58-7388

Fax: +81-285-44-7562

Corresponding Author: Akio Fujimura, MD, Ph.D.

E-mail address: akiofuji@jichi.ac.jp
Structured abstract

Aims: While glucocorticoids are widely used to treat patients with various diseases, they often cause adverse effects such as bone fractures. In this study, we investigated whether the decrease in bone density induced by glucocorticoid therapy was ameliorated by optimizing a dosing-time.

Main methods: Rats were administered with dexamethasone (Dex) orally (1 mg/kg/day) for 6 weeks at a resting or an active period. After the end of the treatment, bone density of femur, biomarkers of bone formation and resorption, and other biomedical variables were measured.

Key findings: Bone density of femur was significantly decreased by the 6-week treatment with Dex, and the degree of decrease in the 14 HALO (hours after light on) dosing group (an active period) was larger than that in the 2 HALO dosing group (a resting period). Although urinary calcium excretion was accelerated by Dex treatment, secondary hyperparathyroidism was not detected. Histomorphometry analysis showed that Dex suppressed bone resorption, which was larger in the 2 HALO than in the 14 HALO groups. These data indicate that Dex equally suppressed bone formation in the 2 and 14 HALO groups, but inhibited bone resorption more in the 2 HALO than in the 14 HALO groups.

Significance: This study shows that the decrease in bone density induced by Dex was changed by its dosing-time.

Key words; chronopharmacology, dexamethasone, osteoclasts, osteoporosis, urinary calcium
Introduction

Glucocorticoids (GCs) are widely used to treat patients with inflammatory disorders such as obstructive airway disease, rheumatoid arthritis and inflammatory bowel diseases. Unfortunately GCs cause several kinds of adverse effects involving hyperglycemia, hypertension and sleep disturbance (Andrews and Walker 1999; Ling et al. 1981; Whitworth et al. 2000). It is well known that the repeated treatment with GCs also increases the risk of bone fractures (Kanis et al. 2004; Van Staa et al. 2000a), which is dose-dependent and occurs rapidly after the initiation of the treatment (Van Staa et al. 2000b). In addition, a recent database analysis showed that the high-dose of oral GCs leads to the increased risk of osteoporotic fracture (De Vries et al. 2007).

Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between the resorption of old or injured bone and the formation of new bone (Dempster 1992). In brief, osteoclast precursors are recruited to a bone surface (activation), and then the newly formed osteoclasts remove both the mineral and organic components of bone matrix (resorption). Osteoblasts and their precursor assemble to refill the resorption cavity. Bone formation is beginning with the deposition of osteoid by the osteoblasts, and then the second stage is mineralization of the organic matrix. Bone remodeling serve two principal functions as follows: renewing the skeleton continuously, and playing a role in mineral homeostasis by transferring calcium and other ions.

It is important to increase the therapeutic effects of drugs and to decrease their adverse effects. Chronotherapy is one of the approaches to achieve these goals by optimizing a dosing-time. We already demonstrated the merits of chronotherapy using several drugs in animals and human subjects (Kitoh et al. 2005; Nozawa et al. 2006; Tsuruoka et al. 2004b;
Dosing-time dependent changes in the pharmacokinetics and/or pharmacodynamics are mainly involved in chronopharmacological phenomenon of drugs (Lemmer 2005).

There are many reports indicating that the circadian rhythmicity is a typical feature of bone metabolism. For example, plasma concentrations of calcium and its regulating hormones (Fraser et al. 1998; Mühlbauer and Fleisch 1995), and markers of bone formation and resorption (Greenspan et al. 1997; Nielsen et al. 1991; Shao et al. 2003; Blumsohn et al. 1994; Bollen et al. 1995) showed circadian rhythms. In addition, the influences of drugs on bone metabolism are reported to be altered by their dosing-time (Schlemmer et al. 1997; Tsuruoka et al. 2002, 2004a, 2004b, 2007). Based on these data, we hypothesized that the decrease in bone density induced by GCs therapy is ameliorated by optimizing a dosing-time.

To address this issue, we examined the influence of GC dosing-time on the decrease in bone density, and evaluated a potential mechanism involving in this phenomenon. In this study, we used dexamethasone, which is a potent synthetic member of the glucocorticoid family.
Materials and Methods

Animal and chemicals

Six-week old male Wistar rats were obtained from Japan SLC Co. (Shizuoka, Japan). They were maintained for more than 2 weeks before the experiment in two rooms under a specific pathogen-free environment and a 12-hour light/dark cycle. The lights were switched on and off at 07:00 and 19:00 in room 1, and at 19:00 and 07:00 in room 2. Rats had free access to standard chow and water. Chow diet used in this study (CE-2, Crea Japan, Tokyo, Japan) contained 1.06% of calcium, 0.98% of phosphorus and 220IU/100mg of Vitamin D. The experiments were performed in accordance with EC Directive 86/609/ECC and the Use and Care of Experimental Animals Committee of Jichi Medical University (Tochigi, Japan). Dexamethasone (Dex) was purchased from Sigma-Aldrich (St Louis, MO), and was suspended in the distilled water. Calcein was obtained from Wako Pure Chemical Industries (Osaka, Japan) and dissolved in 2% NaHCO₃ solution.

Drug dosing and sample collection

After the acclimatization period, rats (n=24) were divided into four groups, and Dex (1 mg/kg p.o.) or vehicle was given by a gastric gavage at two different times [2 or 14 hour after lights on (HALO), Fig. 1] once a day for 6 weeks;

Group 1: vehicle at 2 HALO (n=6), Group 2: Dex at 2 HALO (n=6),
Group 3: vehicle at 14 HALO (n=6), Group 4: Dex at 14 HALO (n=6).

At the final day, rat was administered with 3% of body weight of deionized water at 30 min after dosing and separately placed in a metabolic cage for 4 hours to collect urine sample. Twenty four hours after the last dosing, rat was anesthetized with pentobarbital sodium (50 mg/kg i.p.), and femur and blood were collected. Femur was stored in 70% ethanol, and
serum samples were stored at -80 °C until assay.

For histomorphometry analysis, rats (n=12) were divided into four groups and received the dosing of Dex or vehicle for 6 weeks as described above. Animal was injected with calcein (8 mg/kg i.p.) at 10 and 3 days before the last dosing. Twenty four hours after the last dosing, femur was obtained.

**Measurement of femur bone density**

The bone density of femur was determined by dual energy X-ray absorption (DCS-600A, Aloka, Japan). The scan was performed every 2 mm along the axis of the bone from proximal end, and 18 scans were obtained for each bone. Average of the first proximal 3 scans, middle part of 4 scans, and the last part of 3 scans were regarded as “proximal”, “medial” and “distal”. Average of all scans in bone was shown as “whole”. “Medial” is exclusively cortical bone and “distal” is rich in cancellous bone (Tsuruoka et al. 2002).

**Assays**

Serum osteocalcin concentration was determined by ELISA using a commercialized kit (Biomedical Technologies Inc., Stoughton, MA). Assay was performed according to the instruction manual. The intra- and inter-assay coefficients of variation were better than 7%.

Urinary C-terminal telopeptides of type I collagen (CTx) concentration was measured by ELISA using a commercialized kit (RatLaps™ EIA, Immunodiagnostic systems, Tyne and Wear, UK). Creatinine concentration in urine was measured by an enzyme method (Sekisui Medical Co., Ltd., Tokyo, Japan) by an autoanalyzer. The value of urinary CTx was corrected by creatinine concentration.

Calcium concentration in serum and urine was measured by the orthocresolphthalein
complex method (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) with an autoanalyzer (7170, Hitachi Ltd., Tokyo, Japan).

Serum parathyroid hormone (PTH) concentration was determined by an immunoradiometric assay (Rat PTH IRMA kit, Immunotopics, Inc., San Clemente, CA). Its normal range was 10-40 pg/ml in rats (Tsuruoka, et al. 2002).

**Bone Histomorphometry**

Femur was fixed in 70% ethanol and embedded in glycolmethacrylate without decalcification, and sectioned in 3-mm slices. The sections were stained with Toluidine blue not only to discriminate between mineralized and unmineralized bone, but to identify cellular components. Histomorphometry was performed with semiautomated image analyzing system (OsteoplanII; Carl Zeiss, Thornwood, NY), which was linked to a light microscope at 200-fold magnification. Parameters using the trabecular bone were measured in an area 2.4 mm in length from 0.6-1.2 mm below the growth plate. The following parameters were calculated; trabecular bone volume expressed as a percentage of total tissue volume (BV/TV), percentage of osteoid volume (OV/BS), percentage of osteoid surface (OS/BS), percentage of osteoblasts surface (Obs/BS), number of mature osteoclasts in 10 cm of bone perimeter (N.Oc/B.Prr), percentage of bone surface covered by mature osteoclasts (OcS/BS) and percentage of eroded surface (ES/BS). The thickness of the epiphyseal growth plate cartilage was measured over a length of approximately 2.5 mm in visual areas. The number of proliferative cells and hypertrophic cells was counted in the same visual areas of 0.6 mm inside from epiphyseal side, and were expressed as the average values in six to nine clearly visible columns.
Statistical analysis

Data are shown as the means ± S.D. Comparisons between two groups were done by one-way analysis of variance followed by Bonferroni-Dunn test using StatView (SAS Institute, Cary, NC). \( P < 0.05 \) was considered to be significant.

Results

Influence of Dex dosing-time on bone density of femur

Bone densities of femur in the vehicle-treated rats were not significantly different between the 2 and 14 HALO groups. Repeated treatment with Dex decreased bone density in the 2 and 14 HALO groups (Fig. 2). The parameter in the 14 HALO group was lower than that in the 2 HALO group, especially in “distal”, “medial” and “whole” sections.

Influence of Dex dosing-time on body weight and food intake

The body weights of Dex-treated groups similarly decreased during the experiment period while the parameter in the vehicle-treated groups increased (Fig. 3). Food intake did not significantly differ at any observation points between the vehicle and Dex groups.

Influence of Dex dosing-time on serum osteocalcin concentration

To evaluate the influence of Dex on the bone formation, serum osteocalcin concentration was measured. Serum osteocalcin concentration was significantly decreased by Dex treatment in the 2 and 14 HALO groups (Fig. 4). There was no significant difference in this parameter between the two groups.
Influence of Dex dosing-time on urinary calcium excretion, and serum concentrations of PTH and calcium

The ratio of u-calcium/u-creatinine was significantly increased in the Dex dosing group at 14 HALO, but not at 2 HALO (Fig. 5a). Serum PTH concentrations in the Dex-treated groups elevated, but it did not reach to a statistical significance (Fig. 5b). However, serum calcium concentrations in the Dex-treated groups were significantly higher than those in the vehicle groups (Fig. 5c).

Influence of Dex dosing-time on urinary CTx concentration

To evaluate the influence of Dex on bone resorption, urinary CTx concentration was measured. Urinary CTx concentration tended to be decreased by Dex treatment in the 2 and 14 HALO groups (Fig. 6).

Influence of Dex dosing-time on histomorphometry of trabecular bones of femur

Histomorphometric data did not significantly differ between the vehicle-treated 2 and 14 HALO groups (Table 1a). Bone volume (BV/TV) of the Dex-treated group at 14 HALO was significant lower than that in the vehicle group, while the parameter in the 2 HALO group did not significantly decrease. The percentage of osteoid volume (OV/BS), percentage of osteoid surface (OS/BS), percentage of osteoblasts surface (Obs/BS) and the numbers of mature osteoclasts (N.Oc/B.Prr) were significantly decreased by Dex treatment in both groups. However, bone surface covered by mature osteoclasts (OcS/BS) and consequent eroded surface (ES/BS) were significantly reduced by the treatment with Dex at 2 HALO, but not at 14 HALO.

Histomorphometory data of the epiphyseal growth plate cartilage were shown in Table 1b.
The thickness of growth plate cartilage and the number of proliferative cells per cell column were significantly decreased by the Dex-treated 2 and 14 HALO groups. There were no significant differences in these parameters between the 2 and 14 HALO groups with Dex.

**Discussion**

As a preliminary study, we tested the 0.1, 0.32 and 1 mg/kg of Dex administration for 6 weeks at 2 HALO, and found that only 1 mg/kg of Dex caused a significant decrease in bone density of femur (data not shown). Therefore, we selected the 1 mg/kg of Dex administration for 6 weeks in this study. The preliminary study also showed that body weights were decreased by 0.32 and 1 mg/kg of Dex in rats. These data suggest that the kinds of Dex-induced adverse effects depend on its dose. Although body weights were decreased by Dex treatment at 2 and 14 HALO in this study, food intake did not be decreased by Dex treatment at any observation points, which indicates that Dex-induced bone loss might not be due to a reduced nutrition.

In this study, Dex treatment for 6 weeks reduced bone density of femur in rats. Similar findings were reported as follows; 1) Methylprednisolone reduced the bone mechanical strength due to the decreased bone quantity and quality (Ortoft and Oxlund 1988). 2) Repeated treatment with prednisolone caused osteopenia (Lindgren et al. 1983; Goulding and Gold 1988). However, King et al reported the opposite observation that Dex treatment increased the trabecular bone volume both in intact and parathyroidectomized rats (King et al. 1996). In their study, Dex was given continuously by an osmotic pomp, and a dose of Dex (16.25 µg/rat/day) was small. Although we did not have any definite explanations for the discrepancy of the influence of GCs on bone tissue, it may reside in the differences in the dose of GC and its dosing route.
GCs influence the bone metabolism through the multiple pathways as follows; 1) Direct inhibition on the proliferation of osteoblast (Canalis 1996; Leclerc et al. 2005), 2) Hyperparathyroidism induced by the direct effect on parathyroid gland (Zhang et al. 1993) or the GCs-related increase in urinary calcium excretion (Canalis 1996; Reid 1997), and 3) Direct stimulation (Kaji et al. 1997; Takuma et al. 2003) or inhibition (Dempster et al. 1997; Kim et al. 2006) on the formation of osteoclast.

Dex-induced decrease in bone density in the 14 HALO group was larger than that in the 2 HALO group in this study. To evaluate the mechanism(s) involving in the dosing-time dependent change in the effect of Dex on bone density, we examined the influence of its dosing-time on bone formation. GCs directly impair the proliferation of osteoblasts and subsequently reduce the number and function of mature osteoblasts leading to a decrease in osteocalcin transcription (Canalis 1996; Leclerc et al. 2005). In this study, we measured serum osteocalcin concentration, a biomarker of osteoblasts after 6-week of dosing. The parameter was significantly decreased by the treatment with Dex, but there was no significant difference between the 2 and 14 HALO groups. Therefore, a dosing-time dependent change in the effect of Dex on bone formation might not be involved in the mechanism of this phenomenon.

GCs increase urinary calcium excretion by the direct effect on the kidney, which leads to secondary hyperparathyroidism (Canalis 1996; Reid 1997). Hyperparathyroidism, in turn, activates osteoclasts, which accelerates calcium release from bone tissue and consequently causes bone fracture. Thus, the increased urinary calcium excretion is likely a marker of bone loss induced by Dex. In this study, we determined whether the effect of Dex on the urinary calcium excretion and serum PTH concentration were influenced by its dosing-time. The urinary calcium excretion significantly elevated in the Dex-treated 14 HALO group, and
tended to elevate in the Dex-treated 2 HALO group. These data led us to speculate that Dex dosing at 14 HALO caused hyperparathyroidism, which resulted in the decrease of bone density. However, serum PTH concentration did not elevate by Dex treatment and remained within the normal range between 10-40 pg/ml (Tsuruoka et al. 2002) in the 2 and 14 HALO groups. Thus the role of PTH on a dosing-time dependent effect of Dex might be small, if any.

In general, hypercalcemia decreased PTH secretion. In this study, rats treated with Dex showed hypercalcemia and relatively higher PTH concentration. It is reported that Dex accelerated the secretion of PTH from cultured parathyroid cells and elevated PTH mRNA level (Zhang et al. 1993). Therefore, we think that the direct effect of Dex on parathyroid gland is reflected in the relatively higher PTH observed in this study.

Since serum calcium concentration was significantly elevated by Dex treatment, it was speculated that calcium release from bone tissue might be accelerated. To evaluate a dosing-time dependent effect of Dex on bone resorption, we measured urinary CTx concentration, a marker of bone resorption, and performed the histomorphometry analysis using a fluorescent reagent, calcein. Although all parameters of bone resorption in histomorphometry analysis were decreased by Dex treatment, the degrees of the reduction were significantly larger in the 2 HALO than in the 14 HALO groups. The changes in urinary CTx concentration were comparable with the histomorphometry data. Based on these data, we think that the activity of bone resorption was greater in the 14 HALO than in the 2 HALO groups. Histomorphometry analysis showed that the number of osteoblasts was significantly reduced by Dex in the 2 and 14 HALO groups, which was identical to the result of serum osteocalcin concentration. In addition, the thickness of epiphyseal growth plate cartilage and the number of proliferative cells were similarly decreased by Dex in the 2 and
14 HALO groups. Thus, the Dex-induced suppression on bone formation and epiphyseal cartilage metabolism did not differ between the 2 and 14 HALO groups. These data indicate that a dosing-time dependent change in the effect of Dex on bone resorption is involved in this phenomenon.

The effects of GCs on bone resorption are complex and less clear. Previous in vitro studies demonstrated that GCs increase the formation and activity of osteoclasts (Kaji et al. 1997; Takuma et al. 2003), while other studies showed that GCs inhibit the osteoclasts formation by altering the transcription and enhancing apoptosis (Dempster et al. 1997; Kim et al. 2006). Moreover, a higher dose of methylprednisolone (~ 20 mg/kg) reduced the number of osteoclasts in rats (Hulley et al. 2002; Wang et al. 2002), but a lower dose of Dex did not (King et al. 1996). These findings and present data suggest that a higher dose of GCs inhibits the formation of osteoclasts. However, because pharmacological dose was used in this study, it is unclear whether chronopharmacological phenomena observed in rats are also detected in human subjects treated with Dex. Human study is needed to evaluate a potential dosing-time dependent effect of Dex on bone.

**Conclusion**

The decrease in bone density induced by Dex was larger in the 14 HALO than in the 2 HALO groups. Dex equally suppressed bone formation in the 2 and 14 HALO groups, while it inhibited bone resorption more in the 2 HALO than in the 14 HALO groups, which might cause the dosing-time dependent effect of Dex on bone loss in rats.
Acknowledgement

We thank Mr. Hisashi Murayama (KUREHA SPECIAL LABORATORY Co., Ltd.) for technical assistance in bone histomorphometry analysis.
References


Fraser WD, Logue FC, Christie JP, Gallacher SJ, Cameron D, O'Reilly DS, Beastall GH, Boyle IT, Alteration of the circadian rhythm of intact parathyroid hormone and serum phosphate in women with established postmenopausal osteoporosis. Osteoporosis International 8(2), 121-126, 1998
Goulding A, Gold E. Effects of chronic prednisolone treatment on bone resorption and bone composition in intact and ovariectomized rats and in ovariectomized rats receiving beta-estradiol. Endocrinology 122(2), 482-487, 1988


Hulley PA, Conradie MM, Langeveldt CR, Hough FS. Glucocorticoid-induced osteoporosis in the rat is prevented by the tyrosine phosphatase inhibitor, sodium orthovanadate. Bone 31(1), 220-229, 2002


Kitoh Y, Ohmori M, Araki N, Miyashita F, Ando H, Kobayashi E, Sogawa N, Fujimura A.
Dosing-time-dependent differences in lipopolysaccharide-induced liver injury in rats.  
Chronobiology International 22(6), 987-996, 2005


Tsuruoka S, Nishiki K, Sugimoto K, Fujimura A. Time of day improves efficacy and reduces adverse reactions of vitamin D3 in 5/6 nephrectomized rat. Life Sciences 71(15), 1809-1820, 2002


Ushijima K, Sakaguchi H, Sato Y, To H, Koyanagi S, Higuchi S, Ohdo S. Chronopharmacological study of antidepressants in forced swimming test in mice. Journal


Zhang JX, Fasciotto BH, Cohn DV. Dexamethasone and calcium interact in the regulation of parathormone and chromogranin-A secretion and messenger ribonucleic acid levels in parathyroid cells. Endocrinology 133(1), 152-158, 1993
Table 1a Bone histomorphometry analysis of trabecular bone in femur

<table>
<thead>
<tr>
<th></th>
<th>2 HALO</th>
<th>14 HALO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>Dex</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>19.94 ± 0.51</td>
<td>18.57 ± 1.09</td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>0.417 ± 0.195</td>
<td>0.018 ± 0.003*</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>3.76 ± 1.53</td>
<td>0.14 ± 0.04*</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>4.32 ± 1.88</td>
<td>0.05 ± 0.04*</td>
</tr>
<tr>
<td>N.Oc/B.Prr (/100mm)</td>
<td>136.78 ± 37.06</td>
<td>42.11 ± 9.68**</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>2.81 ± 0.68</td>
<td>0.51 ± 0.08**</td>
</tr>
<tr>
<td>ES/BS (%)</td>
<td>5.70 ± 0.49</td>
<td>1.57 ± 0.31**; #</td>
</tr>
</tbody>
</table>

Mean ± S.D. n=3 in each group

*, P <0.05, **, P <0.01 vs. vehicle; #, P <0.05 vs. 14 HALO Dex
Table 1b Histomorphometry analysis of the epiphyseal growth plate cartilage

<table>
<thead>
<tr>
<th></th>
<th>2 HALO</th>
<th>14 HALO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>Dex</td>
</tr>
<tr>
<td>Thickness (µm)</td>
<td>144.17 ± 15.40</td>
<td>87.15 ± 11.42**</td>
</tr>
<tr>
<td>No. of proliferative</td>
<td>7.65 ± 0.51</td>
<td>4.04 ± 0.06**</td>
</tr>
<tr>
<td>cells per cell column</td>
<td>3.32 ± 0.39</td>
<td>2.93 ± 0.48</td>
</tr>
<tr>
<td>No. of hypertrophic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells per cell column</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± S.D. n=3 in each group

**, P <0.01 vs. vehicle
Captions

Fig. 1 Schematic representation of two reversed lighting regimens to provide two different dosing times at one point. By reversing the lighting condition in two different rooms, dosing at 09:00 approximates the treatment at two circadian stages: 2 and 14 HALO (hour(s) after lights on).

Fig. 2 Influence of Dex dosing-time on bone density of femur

Dex or vehicle was given once daily at 2 HALO or 14 HALO for 6 weeks. □, vehicle at 2HALO; □, Dex at 2 HALO; □, vehicle at 14 HALO; □, Dex at 14 HALO. Mean ± S.D., n=6 in each group *, P<0.05, **, P<0.01 vs. vehicle; #, P<0.05 vs. Dex at 2 HALO

Fig. 3 Influence of Dex dosing-time on body weight and food intake

(a, b); ○, vehicle; □, Dex. (c, d); □, vehicle; □, Dex. Mean ± S.D., n=6 in each group **, P<0.01 vs. vehicle

Fig. 4 Influence of Dex dosing-time on serum osteocalcin concentration

Mean ± S.D., n=6 in each group **, P<0.01 vs. vehicle

Fig. 5 Influence of Dex dosing-time on (a) the ratio of urinary calcium/creatinine, (b) serum PHT and (c) serum calcium concentration

Rats were given with 3% of body weight of deionized water at 30 min after the last dosing, and placed into a metabolic cage for 4 hr to collect urine. Blood sample was obtained at 24 hr after the last dosing. Mean ± S.D., n=6 in each group *, P<0.05; **, P<0.01 vs. vehicle
Fig. 6 Influence of Dex dosing-time on urinary CTx concentration

Urinary CTx concentration was corrected by creatinine concentration. Mean ± S.D., n=6 in each group
Room 1
(Standard lighting)

- Lights off (active period)
- Lights on (resting period)

2 HALO (09:00)
14 HALO

Room 2
(Reversed lighting)

- Lights off (active period)
- Lights on (resting period)

0:00 07:00 19:00 24:00

Fig. 1
Fig. 2

Bone density (mg/cm²)

- distal
- medial
- proximal
- whole

*
**
#

-25-
Fig. 3
Serum osteocalcin (ng/ml)

2 HALO
vehicle | Dex
---|---

14 HALO
vehicle | Dex
---|---

Fig. 4
Fig. 5

(a) The ratio of uCa/uCr (mM/nM)

(b) PTH (pg/ml)

(c) Serum Ca (mg/dL)
Fig. 6

<table>
<thead>
<tr>
<th></th>
<th>2 HALO</th>
<th>14 HALO</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>Dex</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

Urinary CTx (ng/mL creatinine)